

**PRIMERS AND PRIMER SETS FOR USE IN METHODS TO DETECT  
THE PRESENCE OF ACIDOVORAX AVENAE SUBSP. CITRULLI**

**Related Application Information**

5        This application claims priority to U.S. Application No. 60/400,305 filed on August 1, 2002, which is hereby incorporated by reference in its entirety.

**Field of the Invention**

10      The present invention relates to molecular biology, specifically, oligonucleotide primer sequences that can be used in amplification methods, such as PCR and BIO-PCR, to identify the bacterial fruit blotch pathogen, *Acidovorax avenae* subsp. *citrulli* in a test sample.

**Background of the Invention**

15      Bacterial fruit blotch (also known as watermelon fruit blotch) is a serious disease that strikes cotyledons, leaves and young fruit of cucurbits particularly, watermelon and cantaloupe. Bacterial fruit blotch is caused by the bacterium, *Acidovorax avenae* subsp. *citrulli*. Fruit is susceptible to infection during flowering and fruit set and infection does not spread via contact during fruit 20 transit or storage.

25      Symptoms of the bacterial fruit blotch appear on the fruit shortly before harvest, which is often long after the period of infection. Infected areas (lesions) on the rind of watermelon fruit appear water-soaked or oily. Cracks sometimes develop in the more advanced lesions that may contain brown, gummy ooze. Such cracks allow other organisms to enter and cause fruit decay. In melons, the external symptoms on fruit can be very limited, however, the causal agent is invasive and can cause serious degradation of the fruit flesh.

30      Symptoms can also occur on the foliage. The earliest symptoms are water-soaking between the veins on the underside of the seedling cotyledons (seed leaves) (Thomas Isakeit, "Bacterial Fruit Blotch of Watermelon," L-5222,

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Texas Agricultural Extension Service, The Texas A& M University System, 6/99). These areas eventually dry up and die. *Id.* On true leaves, the disease can form distinctive brown, elongated lesions on and next to the veins, which can also appear water-soaked. *Id.* The bacteria also produce brown, circular spots on the leaves although, similar lesions can be caused by a number of other agents. *Id.* Nonetheless, the large lesions on fruit and angular lesions on the true leaves are distinctive for bacterial fruit blotch. *Id.*

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The bacterial fruit blotch pathogen typically spreads to areas where it has not occurred before via seed (seed borne). Environmental conditions play a key role in symptom development and disease severity. *Id.* Rain helps spread bacteria onto developing fruit. Overhead irrigation also contributes to disease development. High temperatures (higher than 90 °F) and high humidity are also a factor.

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Infection of commercial melon fields with bacterial fruit blotch during the 1990's has, in extreme cases, caused losses of about 90% of growers' fields. Such losses have been devastating to the industry. Therefore, there is a need in the industry for sensitive methods of identifying bacterial fruit blotch in seed lots prior to planting to insure against the presence, contamination and spread of the disease in the field.

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### Summary of the Invention

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The present invention relates to oligonucleotide primers comprising sequences of 5'-CGCGCCGACCGAGACCTG - 3' (SEQ. ID NO:1) and 5'-GGGGCACGCCAACATCCT -3' (SEQ. ID NO:2).

The present invention also relates to a primer set comprising the polynucleotide sequence of 5'-CGCGCCGACCGAGACCTG - 3' (SEQ. ID NO:1) and 5'-GGGGCACGCCAACATCCT -3' (SEQ. ID NO:2).

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The present invention further relates to a method of detecting the presence of *Acidovorax avenae* subsp. *citrulli*. in a test sample. The method involves the steps of: (1) providing DNA from a test sample suspected of

containing DNA of *Acidovorax avenae* subsp. *citrulli* or a test sample of bacterial cells or microorganisms suspected of containing DNA of *Acidovorax avenae* subsp. *citrulli*; (2) amplifying a target sequence of DNA from the test sample using a primer set comprising a polynucleotide sequence of: 5'-  
5 CGCGCCGACCGAGACCTG -3' (SEQ. ID NO:1) and 5'-  
GGGGCACGCCAACATCCT -3' (SEQ. ID NO:2) under amplification conditions; and (3) detecting the presence of the amplification product(s) of the target sequence of DNA as an indication of the presence of *Acidovorax avenae* subsp. *citrulli* in the test sample.

10 The present invention further relates to a method of evaluating or monitoring the efficacy of treatments utilized to eliminate *Acidovorax avenae* subsp. *citrulli* from a seed lot. The method involves the steps of: (1) providing a first test sample from a seed lot suspected of containing DNA of *Acidovorax avenae* subsp. *citrulli*; (2) providing DNA from said first test sample or bacterial  
15 cells or microorganisms from the first test sample suspected of containing *Acidovorax avenae* subsp. *citrulli*; (3) amplifying a target sequence of DNA using a primer set comprising: 5'-CGCGCCGACCGAGACCTG -3' (SEQ. ID NO:1) and 5'-GGGGCACGCCAACATCCT -3' (SEQ. ID NO:2) under amplification conditions; (4) detecting the presence of amplification products of the target DNA as an indication of the presence of *Acidovorax avenae* subsp.  
20 *citrulli* in the first test sample; (5) treating the seed lot from which the first test sample was obtained or a portion thereof with a composition to reduce or eradicate *Acidovorax avenae* subsp. *citrulli*; (6) providing a second test sample from the treated seed lot or treated portion thereof; (7) providing DNA from said second test sample or bacterial cells or microorganisms from the second test  
25 sample suspected of containing *Acidovorax avenae* subsp. *citrulli*; (8) amplifying target DNA using a primer set comprising: 5'-  
CGCGCCGACCGAGACCTG -3' (SEQ. ID NO:1) and 5'-  
GGGGCACGCCAACATCCT -3' (SEQ. ID NO:2) under amplification  
30 conditions; (9) detecting the presence of the amplification product(s) of the

target DNA as an indication of the presence of *Acidovorax avenae* subsp. *citrulli* in the second test sample; and (10) comparing the amount of amplification product(s) or absence of amplification products in the second test sample to the amount of amplification products detected in the first test sample in step (4) as an indication of whether or not the treatment has been successful in reducing or eradicating *Acidovorax avenae* subsp. *citrulli* in the seed lot.

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The present invention further relates to a test kit for identifying *Acidovorax avenae* subsp. *citrulli*. The test kit comprises at least one primer set comprising the sequence of: CGCGCCGACCGAGACCTG – 3' (SEQ. ID NO:1) and 5'-GGGGCACGCCAACATCCT –3' (SEQ. ID NO:2).

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The present invention further relates to a test kit for identifying *Acidovorax avenae* subsp. *citrulli*. The test kit comprises a primer set comprising oligonucleotides having the sequence of 5'-CGCGCCGACCGAGACCTG – 3' (SEQ. ID NO:1) and 5'-GGGGCACGCCAACATCCT –3' (SEQ. ID NO:2).

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### **Brief Description of the Figures**

Fig. 1 is a chart of a DNA Extraction Process-Post Seedwash.

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Fig. 2 is a chart of Bacterial Fruit Blotch (BFB) Polymerase Chain Reaction (PCR) Assay for Watermelon Seedlots.

Fig. 3a-3c shows the results of PCR testing for BFB using the primers of the present invention. Specifically, Figs. 3a and 3c show the results of the gel electrophoresis of the amplification products from the PCR. Fig. 3b shows the PCR assay sheet used during the procedure.

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Fig. 4a-4c shows the results of PCR testing for BFB using the primers of the present invention. Specifically, Figs. 4a and 4c show the results of the gel electrophoresis of the amplification products from the PCR. Fig. 4b shows the PCR assay sheet used during the procedure.

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Fig. 5a-5c shows the results of PCR testing for BFB using the primers of the present invention. Specifically, Figs. 5a and 5c show the results of the gel

electrophoresis of the amplification products from the PCR. Fig. 5b shows the PCR assay sheet used during the procedure.

Fig. 6a-6c shows the results of PCR testing for BFB using the primers of the present invention. Specifically, Figs. 6a and 6c show the results of the gel electrophoresis of the amplification products from the PCR. Fig. 6b shows the PCR assay sheet used during the procedure.

Fig. 7a-7c shows the results of PCR testing for BFB using the primers of the present invention. Specifically, Figs. 7a and 7c show the results of the gel electrophoresis of the amplification products from the PCR. Fig. 7b shows the PCR assay sheet used during the procedure.

Fig. 8a-8c shows the results of PCR testing for BFB using the primers of the present invention. Specifically, Figs. 8a and 8c show the results of the gel electrophoresis of the amplification products from the PCR. Fig. 8b shows the PCR assay sheet used during the procedure.

Fig. 9a-9c shows the results of PCR testing for BFB using the primers of the present invention. Specifically, Figs. 9a and 9c show the results of the gel electrophoresis of the amplification products from the PCR. Fig. 9b shows the PCR assay sheet used during the procedure.

#### Sequence Listing

The present application also contains three (3) polynucleotide sequences. The base pairs in these sequences are represented by the following base codes:

	<u>Symbol</u>	<u>Meaning</u>
	A	Adenine
25	C	Cytosine
	G	Guanine
	T	Thymine
	U	Uracil
30	N	A or C or G or T/U

#### Definitions

As used herein, the term "amplification conditions" refers to those conditions that promote annealing and extension of one or more polynucleotide sequences. It is well known in the art that such annealing is dependent in a rather predictable manner on several parameters, including temperature, ionic strength, sequence length, complementarity, and G:C content. For example, lowering the temperature in the environment of complementary polynucleotide sequences promotes annealing. For any given set of polynucleotide sequences, melt temperature, or  $T_m$ , can be estimated by any of several known methods.

Typically, diagnostic applications utilize hybridization temperatures which are close to (i.e. within 10°C.) the melt temperature. Ionic strength or "salt" concentration also impacts the melt temperature, since small cations tend to stabilize the formation of duplexes. Typical salt concentrations depend on the nature and valency of the cation but are readily understood by those skilled in the art. Similarly, high GC content and increased sequence length are also known to stabilize duplex formation because G:C pairings involve 3 hydrogen bonds where A:T pairs have just two, and because longer sequences have more hydrogen bonds holding the sequences together. Thus, high GC content and longer sequence lengths impact the hybridization conditions by elevating the melt temperature.

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Once sequences are selected for a given diagnostic application, the G:C content and length will be known and can be accounted for in determining precisely what hybridization conditions will encompass. Since ionic strength is typically optimized for enzymatic activity, the only parameter left to vary is the temperature. Generally, the hybridization temperature is selected close to or at the  $T_m$  of the primers or probe. Thus, obtaining suitable hybridization conditions for a particular primer/probe set is well within ordinary skill of one practicing this art.

The terms "oligonucleotide" and "primer" are used interchangeably herein.

5           The term "test sample" refers to bacterial cells, microorganisms or extracted DNA. The test sample can be a sample suspected of containing bacterial cells or microorganisms and thus, the DNA of bacterial cells or microorganisms, or a test sample containing extracted DNA.

10          As used herein, the term "polynucleotide" refers to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. This term refers only to the primary structure of the molecule. Thus, the term includes double- and single-stranded DNA, as well as double- and single-stranded RNA. It also includes modifications, either by methylation and/or by capping, and unmodified forms of the polynucleotide.

15          As used herein, the term "primer" refers to a polynucleotide that is approximately between about fifteen (15) to twenty-five (25) nucleotides in length. A primer is capable of acting as a point of initiation of synthesis on a polynucleotide sequence when placed under appropriate conditions. The primer will be completely or substantially complementary to a region of the target polynucleotide sequence to be copied. Therefore, under conditions conducive to hybridization, the primer will anneal to the complementary region of the target sequence. Upon addition of suitable reactants, including, but not limited to, a polymerase, nucleotide triphosphates, etc., the primer is extended by the polymerizing agent to form a copy of the target sequence. The primer may be single-stranded or alternatively may be partially double-stranded.

20          As used herein, the term "probe" refers to a polynucleotide that is at least eight (8) nucleotides in length and which forms a hybrid structure with a target sequence, due to complementarity of at least one sequence in the probe with a

sequence in the target region. The polynucleotide regions of the probe can be composed of DNA and/or RNA and/or synthetic nucleotide analogs. Preferably, the probe does not contain a sequence complementary to the sequence(s) used to prime for a target sequence during the polymerase chain reaction.

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As used herein, the term "label" or "labeled" refers to any atom or moiety which can be used to provide a detectable (preferably, quantifiable) signal, and which can be attached to a polynucleotide, primer or probe. A wide variety of labels and conjugation techniques, including direct and indirect labeling, are known and are reported extensively in both the scientific and patent literature. Examples of labels that can be used include radionucleotides, enzymes, substrates, cofactors, inhibitors, fluorescent moieties, chemiluminescent moieties, magnetic particles, and the like. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

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As used herein, the term "plant" includes plant cells, plant protoplasts, plant cell tissue cultures from which cucurbitaceae plants can be regenerated, plant calli, plant cell clumps, and plant cells that are intact in plants, or parts of plants, such as embryos, pollen, ovules, flowers, leaves, seeds, roots, root tips and the like.

#### **Detailed Description of the Invention**

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In one embodiment, the present invention provides novel oligonucleotide primers and primer sets. These primer and primer sets described herein can be used in amplification methods (such as polymerase chain reaction and BIO-PCR) and packaged into kits for use in amplification methods for the purpose of detecting *Acidovorax avenae* subsp. *citrulli* in a test sample. Additionally, these primers and primer sets can be used in amplification methods (such as polymerase chain reaction and BIO-PCR) to evaluate or monitor the efficacy of

treatments being used to eliminate *Acidovorax avenae* subsp. *citrulli* from a seed lot. These oligonucleotide primers are designated as SEQ. ID NO:1 and SEQ ID NO:2 and their polynucleotide sequences are as follows: (1) (forward) 5'-CGCGCCGACCGAGACCTG - 3' (SEQ. ID NO:1); and (2) (reverse) 5'-GGGGCACGCCAACATCCT - 3' (SEQ. ID NO:2). When the primers designated in SEQ ID NO:1 and SEQ ID NO:2 are used in PCR, an amplification product of approximately 232 base pairs is obtained for *Acidovorax avenae* subsp. *citrulli*.

The primers of the present invention can be prepared using techniques known in the art, including, but not limited to, cloning and digestion of the appropriate sequences and direct chemical synthesis. Unlike other primers known in the art, the primers of the present invention are sensitive in detecting the presence of *Acidovorax avenae* subsp. *citrulli* (*Aac*) in a test sample. For example, the primers of the present invention can identify the presence of *Acidovorax avenae* subsp. *citrulli* in a single seed in a sample of about 5,000 seeds.

Chemical synthesis methods that can be used to make the primers of the present invention, include, but are not limited to, the phosphotriester method described by Narang et al., *Methods in Enzymology*, 68:90 (1979), the phosphodiester method disclosed by Brown et al., *Methods in Enzymology*, 68:109 (1979), the diethylphosphoramidate method disclosed by Beaucage et al., *Tetrahedron Letters*, 22:1859 (1981) and the solid support method described in U.S. Patent No. 4,458,066. The use of an automated oligonucleotide synthesizer to prepare synthetic oligonucleotide primers of the present invention is also contemplated herein. Additionally, if desired, the primers can be labeled using techniques known in the art.

As mentioned briefly earlier, the oligonucleotide primers and primer sets of the present invention can be used in an amplification method to identify the bacterial pathogen *Acidovorax avenae* subsp. *citrulli*. Additionally, these primers and primer sets can be used in amplification methods (such as

polymerase chain reaction and BIO-PCR) to evaluate or monitor the efficacy of treatments being used to eliminate *Acidovorax avenae* subsp. *citrulli* from a seed lot. The amplification methods that can be used include polymerase chain reaction (PCR), which is described in U.S. Patent Nos. 4,683,195 and 4,683,202, or BIO-PCR which is described in U.S. Patent No. 6,410,223 and Schaad et al., *Phytopath.*, 85:243-248 (1995).

Generally, in PCR, a target polynucleotide sequence is amplified by reaction with at least one oligonucleotide primer or pair of oligonucleotide primers. The primer(s) hybridize to a complementary region of the target sequence and a DNA polymerase extends the primer(s) to amplify the target sequence. If the PCR test is designed properly, a DNA fragment of one size dominates the reaction products (the target polynucleotide sequence which is the amplification product). The amplification cycle is repeated to increase the concentration of the single target polynucleotide sequence. The amplification products can be separated using methods known in the art such as gel electrophoresis. Southern blot analysis or blot analysis can be used to verify that the amplification product is in fact the target polynucleotide sequence. Alternatively, a far more stringent way to verify the product is to sequence it.

Alternative methods of conducting PCR with various combinations of primers (i.e. from 1 to 3 primers) are known in the art and are described in Maniatis, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor, N.Y. (1989). These methods include asymmetric PCR, PCR using mismatched or degenerate primers, reverse transcriptase PCR, arbitrarily primed PCR (Welsh et al., *Nucleic Acids Res.* 18:7213-7218 (1990)), RAPD PCR, IMS-PCR (Islam et al., *J. Clin. Micro.*, 30:2801-2806 (1992); and multiwell PCR. Additionally, amplification methods using a single primer, such as described by Judd et al., *Appl. Env. Microbiol.*, 59:1702-1708 (1993) are also contemplated herein.

BIO-PCR combines biological preamplification of the PCR target organism with enzymatic amplification of the PCR target. The advantages of BIO-PCR over the standard PCR assay, include, the detection of live cells only,

a 100-1000 fold increase in sensitivity, and elimination of PCR inhibitors associated with plant samples thereby eliminating false negatives. Methods for conducting BIO-PCR are described in U.S. Patent 6,410,223.

In another embodiment, the present invention relates to a method of detecting *Acidovorax avenae* subsp. *citrulli* in a test sample. This method uses the amplification methods previously described herein. Generally, DNA amplification involves the following steps: (a) providing a test sample comprising bacterial cells or microorganisms or extracted DNA for standard PCR or bacterial cells or microorganisms amplified by growing on an agar medium for BIO-PCR that is suspected of containing DNA from *Acidovorax avenae* subsp. *citrulli*; (b) amplifying a target sequence of the DNA with the primers of the present invention under amplification conditions to provide DNA amplification products carrying the selected target DNA sequence; and (c) detecting the presence of the DNA amplification products as an indication of the presence of live *Acidovorax avenae* subsp. *citrulli* in the test sample.

As discussed briefly above, the test sample can be bacterial cells or microorganisms or extracted DNA. The extracted DNA can be from any biological sample, such as from a plant, bacterial cells, microorganism, etc. Extraction of DNA from a biological sample can be conducted using routine techniques known in the art.

If PCR is to be used to detect *Acidovorax avenae* subsp. *citrulli* in a test sample, the test sample can be provided in an aqueous buffer formulated with an effective amount of Mg<sub>2</sub> co factor. Additionally, the reaction is conducted with an effective amount of a DNA polymerase (such as Taq DNA polymerase, which can be in the form of native purified enzyme or a synthesized form such as AMPLITAQ (which is available from Applied Biosystems, Foster City, CA)), an effective amount of deoxynucleotide triphosphates (i.e., dATP, dCTP, dGTP, and dTTP) or nucleotide analogs (dNTPs) as a nucleotide source in a saturating concentration and an effective amount of one or the pair of primers described herein. Alternatively, PCR premixes, which contain buffer, dNTPs and Taq

polymerase as a premixed reagent can be used. If such a premix were used, an effective amount of one or the pair of primers described herein would need to be added.

Methods for setting up a PCR reaction are well known to those skilled in the art. Specifically, a buffered reaction mixture is prepared that contains the DNA substrate to be amplified, the primer or primers, Mg<sup>+2</sup> cofactor, dNPTs and Taq polymerase. Prior to the PCR cycling, a single step is usually performed to denature the DNA substrate. This step usually is performed between about 92°C to about 95°C for about 2 to about 10 minutes. Following this initial 5 denaturation step, a cycling process occurs to amplify the target sequence. The number of cycles typically varies between about 25 to about 40, and there normally are 2 or 3 steps in each cycle. During each cycle, three things happen. The first step denatures both the original DNA substrate, and any nascently formed products. This step requires between 10 seconds and several minutes at 10 about 92°C to about 95°C. Following the denaturation step, the primers have to anneal to their complementary regions of the target sequence, and then the DNA 15 polymerase needs to extend the primers to synthesize the desired product. Depending on the melting temperature of the primers, this can be accomplished in one, or two steps. After the primer(s) are annealed to the target, the optimum 20 temperature to extend these primers using Taq polymerase is about 72°C. If the T<sub>m</sub> of the primers is close to this temperature, both the annealing and synthesis steps can be performed at the same time, resulting in a two-step cycle 25 (denaturation followed by annealing/synthesis). If the T<sub>m</sub> of the primers is much lower than about 72°C, it is common to have a three step cycle. In a three step cycle, there is a denaturation step, a primer annealing step, and a extension step. Each of these steps can be between 10 seconds and 2 minutes. An example of a 3 step reaction would be 30 seconds at about 95°C, 30 seconds at about 55°C, and 60 seconds at about 72°C.

If *Acidovorax avenae* subsp. *citrulli* is present in the test sample, a single amplification product results. This amplification product is about 200-300 base pairs in length, preferably about 232 base pairs in the length, whose termini are defined by the oligonucleotide primer(s) of the present invention and whose length is defined by the distance between the two primers or the length of time of the amplification reaction. This polynucleotide sequence (i.e., the amplification product) then serves as a template for the next reaction.

The amplification product can be separated from the reaction mixture and analyzed. The amplified polynucleotide sequence can be analyzed using techniques known in the art, such as gel electrophoresis or other techniques known in the art. Additionally, the amplified polynucleotide sequence can be directly or indirectly labeled using techniques known in the art. A far more stringent way to verify the amplification product is to sequence the product.

To verify the identity of the amplification product, a Southern blot assay can be conducted. In a Southern blot assay, the amplification products are separated by electrophoresis, transferred to a membrane (i.e. nitrocellulose, nylon, etc.), reacted with an oligonucleotide probe or any portion of the DNA sequence of interest. The probe is then modified to enable detection. The modification methods can be the incorporation of a radiolabeled nucleotide or any number of non-radioactive labels (such as biotin).

The oligonucleotide probe used in the Southern blot assay is derived from the genomic DNA sequence of *Acidovorax avenae* subsp. *citrulli* and hence is specific for DNA from *Acidovorax avenae* subsp. *citrulli*. The probe used in the Southern blot assay can be prepared using routine, standard methods. For example, the probe can be isolated, cloned and restricted using routine techniques known in the art or can be made using the chemical synthesis methods described previously herein.

Alternatively, the amplification products can be detected using dot blot analysis. Dot blot analysis involves adhering an oligonucleotide probe (such as the one described previously) to a nitrocellulose or solid support such as, but not

limited to, a bead (such as, but not limited to, polystyrene beads, magnetic beads or non-magnetic beads, etc), walls of a reaction tray, strips (such as, but not limited to nitrocellulose strips), test tube. The sample containing the labeled amplification product is added, reacted, washed to removed unbound sample, and a labeled, amplified product attached to the probe is visualized using routine techniques known in the art.

The detection of the amplification product in the sample provides evidence of the presence of *Acidovorax avenae* subsp. *citrulli* in the test sample. If BIO-PCR is used, the method described herein can be used to identify the presence of viable cells of *Acidovorax avenae* subsp. *citrulli*. As described in U.S. Patent No. 6,146,834, detection utilizing BIO-PCR can be enhanced further when combined with the “TaqMan” detection system which is described in Holland et al., *Proc. Natl. Acad. Sci. USA*, 88:7276-7280 (1991). TaqMan is a PCR based assay that is modified to contain a “fluorescent reporter” and a “fluorescent quencher” pair at the 5' and 3' termini. Typically, this probe about is 8 to about 25 nucleotides in length, which positions the quencher close enough to the reporter to quench the reporter signal. If this probe anneals to the target sequence during PCR, the 5' to 3' exonuclease activity of the DNA polymerase causes the reporter and quencher to be untethered, resulting in a recordable fluorescent signal that is proportional to the amount of target signal being amplified.

In yet another embodiment, the present invention relates to a method of evaluating or monitoring the efficacy of treatments being utilized to eliminate *Acidovorax avenae* subsp. *citrulli* from a seed lot. The method involves obtaining a first test sample from a seed lot suspected of containing *Acidovorax avenae* subsp. *citrulli*. Once this first test sample is obtained, DNA is extracted from the test sample using routine techniques known in the art or bacterial cells or microorganisms from the test sample suspected of containing DNA of *Acidovorax avenae* subsp. *citrulli* are supplied for use in an amplification method (i.e., PCR or BIO-PCR). The extracted DNA or bacterial cells or

microorganisms suspected of containing DNA of *Acidovorax avenae* subsp. *citrulli* are then amplified (i.e. using PCR or BIO-PCR) using primers having the sequence of 5'- CGCGCCGACCGAGACCTG – 3' (SEQ. ID NO:1) and 5'- GGGGCACGCCAACATCCT –3' (SEQ. ID NO:2). Then, the presence of the amplification product(s) of the target DNA is then detected using the techniques described herein. The presence of the amplification product(s) of the target DNA is confirmation of the presence of *Acidovorax avenae* subsp. *citrulli*.

If the presence of *Acidovorax avenae* subsp. *citrulli* in the first sample is confirmed, then the seed lot from which the first sample was obtained or a portion of that seed lot can be treated with a composition to reduce or eradicate *Acidovorax avenae* subsp. *citrulli*. Such compositions are well known in the art and include, but are not limited to, peroxyacetic acid. After a sufficient period of time has elapsed to allow such treatment to take effect, a second test sample is taken from the treated seed lot or treated portion thereof. As with the first test sample, DNA is extracted from the second test sample using routine techniques known in the art or bacterial cells or microorganisms from the second test sample are supplied for use in an amplification method. The extracted DNA or cells or microorganisms are then amplified (i.e. using PCR or BIO-PCR) for target DNA *Acidovorax avenae* subsp. *citrulli* of using primers having the polynucleotide sequence of 5'- CGCGCCGACCGAGACCTG – 3' (SEQ. ID NO:1) and 5'-GGGGCACGCCAACATCCT –3' (SEQ. ID NO:2) under amplification conditions. Then, the presence of the amplification product(s) of the target DNA is then detected using the techniques described herein. Finally, the amount of the amplification product(s) or absence of amplification product(s) in the second test sample is compared to the amount of amplification product(s) in the first test sample. If the amount of amplification product(s) is less than the amount of amplification product(s) in the first test sample or if there is no amplification product(s) in the second test sample, this provides evidence that the treatment protocol being utilized to eliminate *Acidovorax avenae* subsp. *citrulli* seed lot is efficacious. If the amount of amplification

product(s) is more than the amount of amplification product(s) in the first test sample, this provides evidence that the treatment protocol being utilized to eliminate *Acidovorax avenae* subsp. *citrulli* seed lot is not efficacious.

In yet another embodiment, the present invention relates to a test kit for identifying *Acidovorax avenae* subsp. *citrulli*. More specifically, the oligonucleotide primers and primer sets of the present invention can be packaged into kits for use in amplification methods for identifying *Acidovorax avenae* subsp. *citrulli* in a test sample. The kits will contain the primers in a premeasured or predetermined amount, as well as other suitably packaged reagents and materials, in separate suitable containers, need for the particular amplification method. For example, the kit may contain standard buffers, solid supports, enzymes, substrates, instructions, etc., for identifying *Acidovorax avenae* subsp. *citrulli* in a test sample.

By way of example, and not of limitation, examples of the present invention shall now be given.

Example 1: Development of the Primers of the Present Invention

Primers (identified as RST 90 and RST 91) supplied by R. Stall (University of Florida) were used in an amplification reaction to amplify *Acidovorax avenae* subsp. *citrulli*. A 380 base pair PCR product was obtained, cloned and sequenced, and the sequence of the product was used to develop the primers of the present invention using routine techniques known in the art such as those described in *Current Protocols in Molecular Biology*, Ausubel, Brent, Kingston, Moore, Seidman, Smith and Struhl, editors, John Wiley & Sons (1994).

More specifically, the PCR product was resolved on an agarose gel, and excised using a razor blade. Comparison of the product with molecular mass markers provided an estimate of the size of the product around 400 base pairs. Excised DNA was purified from the agarose by crushing the agarose into small pieces and centrifuging the slurry through a small column packed with glass wool.

Purified DNA was ligated into the plasmid vector pT7 Blue using the Perfectly Blunt PCR Cloning Kit, according to the Manufacturer's instructions (Novagen Inc., Milwaukee, WI 53288), and transformed into *Escherichia coli* (Novagen Blue). After transformation, aliquots were spread onto media plates according to the Novagen kit instructions and colonies were allowed to grow by incubating the plates overnight at 37°C.

*E. coli* clones harboring the pT7 Blue plasmid with the insert DNA were selected by color selection, and then by size selection by running digested DNAs on an agarose gel. One of these clones was selected, and renamed pSVS1224. DNA was prepared from pSVS1224 using standard methods, and the inserted DNA was sequenced using chemistry from Applied Biosystems, and resolved on an ABI377 automated sequencer (Applied Biosystems, Foster City, CA).

The resulting sequence, shown below, was imported into the LASERGENE molecular biology software package (LASERGENE, Madison, WI). The primer select module of LASERGENE was used to design new PCR primers, referred to as WFB1 (SEQ ID NO:1) and WFB2 (SEQ ID NO:2).

ACATGGCCAC GGCGATGAGG ATGCCCGGCG CGAACGCCGGC  
GATGAAGAGC GCGCCGACCG AGACCTGCTC GTCCTGCAGC  
20 GCGTAGATGA TCATGATGAT CGAGGGCGGG ATGATGGGCC  
CCACGATGGC CGTGCTGGCG GTGAGCGCGG CGGCGTATGC  
GCGCGAGTAG CCCGCCTTGT CCATCATCTT GACCATCATC  
GAGCCGGGCC CCGCCCGCTC GGCCAGGGCC GAGCCCGAGA  
TGCCCGAGAA CAGCGTGAGC GACAGGATGT TGGCGTGCC  
25 CAGGCCCGCG CGCAGGTGGC CCACGAACTG CGCGGCGAAG  
CGCAGCAGCA CCACGGTGAG CGCGCCGCCG GACATGATCT  
CGGCCGCGAG GATGAAGAAG GCACGGCCAT CG (SEQ ID NO:3)

#### Example 2a: Protocol for Preparing Seed Samples Prior to PCR

A culture of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) is initiated on YDC agar (hereinafter referred to as “YDC”) and incubated at 28°C. If timing is such that a culture cannot be started two days ahead of the test (assay) date, then one can be initiated one day ahead with heavy streaking on YDC. The *Xcv* is included in the test as an added control to insure that the test is performing correctly. The test can be conducted without the inclusion of the *Xcv*. Initiate 3-4 tubes of Nutrient Broth with a viable culture of *Acidovorax avenae* subsp. *citrulli* (*Aac*) the day before the test is to be run. Place the tubes on a shaker at 37°C at 200 rpm. Count out two (2) samples of 5,000 seeds of the seeds to be used as the negative and positive control and place in Ziplock bags. Label the bags with the appropriate sample numbers and record.

Count out the appropriate number of samples (of seed) to be tested. The number to be tested by PCR can range from about 10,000 to about 30,000 seeds with the balance being done by growout. The inspection lot and batch number are recorded on a record sheet. A separate disposable container is used to weigh each lot. Any positives obtained in the PCR test are retested at 1X in a greenhouse grow-out test.

Each subsample of 5,000 seeds is placed in a 1.0 gallon freezer Ziplock bag. Once sealed, this bag is placed inside another Ziplock bag. Both bags are labeled with the sample (batch) number.

Prepare the amount of PABST buffer needed for the number of samples to be tested. PABST is Phos-Tween (See the enclosed protocol for making Phos-Tween in Example 2d) ascorbic acid and potassium metabisulfite. PABST buffer is made fresh immediately before use (See enclosed protocol for making PABST buffer in Example 2d).

The PABST buffer is prepared by adding the ascorbic acid and potassium metabisulfite to two liters of Phos-Tween. After the chemicals are dissolved, the remaining volume of Phos-Tween is added. The pH is adjusted to about 6.5 using concentrated NaOH (approximately about 5 mls/liter of PABST).

A suspension of *Xanthomonas campestris* pv. *vesicatoria* is prepared in a 50 ml disposable sterile centrifuge tube in Phos-Tween. Enough material is used from the single colonies on a YDC culture to prepare a faintly turbid suspension. The suspension is vortexed to ensure that all the culture material is in solution.

5       The optical density (OD<sub>620</sub>) of the suspension is measured and adjusted to about 0.045 to about 0.055.

Vortex the nutrient broth suspension of *Aac* prepared previously, and add up to about 9 mls of Phos-Tween. The concentration is adjusted so that the OD<sub>620</sub> is between about 0.095 and about 0.105. The Phos-Tween is used as a reference for the spectrophotometer. A 1:10 serial dilution of the OD adjusted *Aac* suspension to 10<sup>-7</sup> in 9 ml tubes of Phos-Tween.

10      The required amount of PABST is added to the seed samples in Ziplock bags. The bags are sealed and placed upright in plastic tubs. After the PABST is added to all bags, one ml of the previously made *Xcv* suspension is added to each of the bags in the assay with the exception of the positive control. The bags are closed tightly.

15      About 2.3 ml of the 10<sup>-3</sup> *Aac* dilution tube made previously is added to the positive control. This is now the *Aac* positive control. The bag is sealed and all bags in the tubs are placed into the shaker. The bags are shaken for about one hour at 150 rpm at room temperature.

20      About 0.2 mls of the 10<sup>-6</sup> and 10<sup>-7</sup> dilutions of *Aac* are spread-plated onto 8 Nutrient Agar for each dilution. About 0.2 mls of the 10<sup>-6</sup> dilution are spread onto 4 Tween plates. The spread-plates are incubated for 24-48 hours at about 28°C. After incubation, the colony numbers are counted for calculation of the actual number of cells added to the control seed.

25      The inner bag of seed from the Ziplock bags is removed and placed into funnels. Two or three small vertical slits are cut into one of the bottom corners and the liquid rinsate is collected by allowing it to drain into a centrifuge bottle (such as a 250 ml centrifuge bottle). The seed is discarded. The centrifuge bottles are balanced for centrifuging. If necessary, a small amount of PABST

can be added. Centrifuge the rinsate, such as in a GSA rotor in a Sorvall RC-5B centrifuge, for about 5 minutes at 2500 rpm to pellet the soil and debris. Transfer the supernatant into a clean centrifuge bottle, such as a 250 ml centrifuge bottle. Decant slowly so that if the pellet is soft pieces of the pellet will not accompany the supernatant. If necessary, leave a few milliliters of solution in the bottle to avoid particulates. Centrifuge the filtrate, such as in a GSA rotor in a Sorval RC-5B centrifuge), for about 15 minutes at 5,600 rpm to pellet the bacteria. Discard the supernatant carefully and preserve the pellet. If necessary, leave a few milliliters of solution in a bottle to avoid discarding the soft pellet pieces.

5                   About 0.5 grams of polyvinylpyrrolidone (PVPP; see protocol in Example 2d) is added to each centrifuge bottle. Add the contents of a 5 ml tube of Phos-Tween into the centrifuge bottle and resuspend the pellet. This suspension is incubated at room temperature for about 30 minutes. After about 30 minutes, the suspension is poured through a Miracloth (Calbiochem, San Diego, California) and collected in a centrifuge tube. The resulting liquid fraction is then

10                  centrifuged for about 15 minutes at 7,000 rpm using a SA600 rotor to pellet the bacterial cells. Discard the supernatant. Resuspend the pellet in about 1.3 mls of Phos-Tween, although less can be used if liquid is retained in the tube. This final suspension is now ready for DNA extraction.

15                  

20                  Example 2b: DNA Extraction

The final suspension from Example 2a is centrifuged using an Eppendorf 5415C centrifuge and rotor at about 16,000 g for about 2 minutes. The supernatant is discarded and the pellet resuspended in about 582 µl of TE buffer (See Example 1d for protocol for TE buffer). Add about 15 µl of 20% sodium dodecyl sulfate (SDS) and about 3 µl of Proteinase K (See Example 2d) and the suspension is incubated at 37°C for about 1 to about 1.5 hours. Add about 100 µl of 5M NaCl and 80 µl of hexadecyltrimethylammonium bromide (CTAB) (See Example 2d). The suspension is incubated in a water bath at 65°C for about 15 minutes. Add about 770 µl of chloroform-isoamyl alcohol (24:1) (See Example 2d). The suspension is mixed for about 15 minutes then centrifuged

for about 5 minutes at about 16,000 g using an Eppendorf 5417C centrifuge and rotor. The aqueous supernatant is discarded and placed into a 2 ml tube. About 500  $\mu$ l of phenol/chloroform/isoamyl alcohol (25:24:1) is added. The suspension is mixed for about 15 minutes and then centrifuged for about 15 minutes at about 16,000 g using a an Eppendorf 5417C centrifuge and rotor. About 550  $\mu$ l of supernatant is transferred to a 1.5 ml tube. About 550  $\mu$ l of cold isopropyl alcohol is added. The suspension is mixed and stored for at least about four (4) hours at about -80°C.

The samples are removed from storage, thawed and centrifuged for about 30 minutes at about 16,000 g using a an Eppendorf 5417C centrifuge and rotor. Discard the supernatant and wash the pellet with about 500  $\mu$ l of 70% ethanol. Centrifuge the sample for about 10 minutes at about 16,000 g using an Eppendorf 5417C centrifuge and rotor. Remove the supernatant and dry the pellet in a vacuum dessicator for about 10 minutes. Redissolve the pellet in about 100  $\mu$ l of TE buffer and incubate the samples in a 65°C water bath. Vortex and mix the samples well. Make a 1:50 dilution of the resuspended DNA in water and run about 50  $\mu$ l through a BioRad Microspin column.

#### Example 2c: PCR

Load 5  $\mu$ l of the DNA suspension from Example 2b into each of the two tubes of PCR reaction mixture. Each reaction well contains: 5  $\mu$ l of 10X PCR buffer (Perkin Elmer), 8  $\mu$ l of 1.25 mM dNTPs, 3.3  $\mu$ l of MgCl<sub>2</sub>, 1  $\mu$ l at 5 pmol/ $\mu$ l of each of SEQ ID NO:1 and SEQ ID NO:2, 0.25 of *Taq* polymerase, and 26.45  $\mu$ l of water. Each sample tube is run in a PCR thermocycler (Perkin Elmer GeneAmp PCR System 9600 or an Applied Biosystems GeneAmp PCR System). The amplifications were conducted under the following conditions: initial DNA denaturation for 10 minutes at about 93°C, followed by a cycling step of about 95°C for 1 minute, 62.1°C for 30 seconds, 72°C for about 30 seconds. The PCR cycle is repeated for about 35 cycles. After cycling, there is a final extension step of about 5 minutes at 72°C followed by storage of the products at 4°C until they are removed from the PCR machine. To detect a

product, 10  $\mu$ l of loading dye (defined below) is added to each reaction well. About 16  $\mu$ l of PCR reaction is added to an electrophoresis gel containing 2% Seakem LE (defined below) agarose in 0.5X TBE buffer (see Example 2d) and electrophoresed. as described in Maniatis et al., *Molecular Cloning, a Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y. (1989). The gel consists of 2% Seakem LE in TBE buffer (See Example 2d). The gel is run at 80 or 120 volts depending on the size of the gel.

5 Example 2d: Buffers and Reagents

10 Protocol for making Phos-Tween and 1L of PABST

In 200.0 ml MM H<sub>2</sub>O dissolve:

Potassium Phosphate (K <sub>2</sub> HPO <sub>4</sub> · 3H <sub>2</sub> O)	1.96 g
Potassium Phosphate (KH <sub>2</sub> PO <sub>4</sub> )	0.994 g
Tween 20	0.20 ml

Bring volume up to 1.0 L

15 Adjust pH = 6.5

(using 5M HCl.)

At this point this mixture is known as 'Phos-Tween'

Sterilize

Before using add:

L -Ascorbic Acid (C <sub>6</sub> H <sub>8</sub> O <sub>6</sub> )	17.6 g
Potassium Metabisulfite (K <sub>2</sub> O <sub>5</sub> S <sub>2</sub> )	2.2 g

Adjust pH= 6.5

(using NaOH)

This formula is now referred to as PABST.

- 25 • 5XTBE Stock: Tris-Borate-EDTA Buffer (pH=8.35)

Dissolve the following reagents in 800 mls MQ-water in a 2-liter flask on a stir plate;

54.0 g Trizma Base (Sigma Aldrich ("Sigma"), St. Louis, MO, Cat. #T8524)

30 27.5 g Boric Acid (Sigma Cat. # B-6768)

20 mls 0.5M (Ethylenedinitrilo) tetraacetic acid disodium salt (EDTA)  
stock pH=8.0

Adjust the total volume to 1.0 liter

<b>Handling</b>	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				
NO	XX	XX	XX	XX

- 10X Tris-Acetate-EDTA buffer stock

Dissolve the following in 800 mls MQ-water in a 2 liter flask on a stir plate;

48.4g Trizma Base (Sigma cat. # T-8524)

11.42 mls glacial acetic acid (Sigma cat. # A6283)

20 mls 0.5M EDTA

Adjust the volume to 1.0 liter

Because this stock is highly concentrated it may need extra time on the stir plate to completely come to solution.

<b>Handling</b>	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				
NO	XX	XX	XX	XX

20

- 1X Tris-Acetate-EDTA (TAE) Buffer

Mix 100 mls of 10X TAE and 900 mls of MQ-water.

<b>Handling</b>	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES			XX	
NO	XX	XX		XX

25

- **PABST buffer:** BFB-PCR seed wash buffer

Dissolve 17.6 g Ascorbic Acid (Sigma cat. # A-7506) and 2.2 g

Potassium Metabisulfite (Sigma cat. # P-2522) in 1.0 liter of Phos

30

Tween buffer. Adjust the pH to 6.50 with Saturated NaOH.

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES	XX			5, 9 & 15 mls/tube
NO		XX	XX	Also 2 liter bottler

- 5            • **5.0M NaCl:** Sodium Chloride  
                 Dissolve 29.22 g of NaCl (available from VWR International, West Chester, PA, cat. # VW 6430-7) in 80 mls of MQ water. Adjust the volume to a total of 100 mls.
- 10           • **CTAB/NaCl:** Hexadecyltrimethylammonium Bromide/Sodium Chloride solution.  
                 Dissolve 4.1g NaCl (available from VWR International cat. # VW 6430-7) and 10 g CTAB (Sigma cat. # H-6269) in 100 mls of MQ water. Mix the reagent by spinning on a stir plate with mild heat until the crystals are completely dissolved.
- 15           • **TE buffer:** Tris-EDTA  
                 Dissolve 0.394 g Trizma Hydrochloride (Sigma cat. # T-7419) and 1.0 mls of 0.25M EDTA stock in 250 mls MQ water. Adjust the pH to 8.0 using 1.0 M NaOH.
- 20           • **70% Ethanol**
- 25           • **5.0M NaCl:** Sodium Chloride  
                 Dissolve 29.22 g of NaCl (available from VWR International, West Chester, PA, cat. # VW 6430-7) in 80 mls of MQ water. Adjust the volume to a total of 100 mls.
- 30           • **CTAB/NaCl:** Hexadecyltrimethylammonium Bromide/Sodium Chloride solution.  
                 Dissolve 4.1g NaCl (available from VWR International cat. # VW 6430-7) and 10 g CTAB (Sigma cat. # H-6269) in 100 mls of MQ water. Mix the reagent by spinning on a stir plate with mild heat until the crystals are completely dissolved.
- 35           • **TE buffer:** Tris-EDTA  
                 Dissolve 0.394 g Trizma Hydrochloride (Sigma cat. # T-7419) and 1.0 mls of 0.25M EDTA stock in 250 mls MQ water. Adjust the pH to 8.0 using 1.0 M NaOH.
- 40           • **70% Ethanol**

Add 70.0 mls of 100% ethanol to 30.0 mls of MQ water in the hood and invert to mix.

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				12.0 mls/vial
NO	XX	XX	XX	

5

- **20% SDS:** Sodium Dodecyl Sulfate

10

(This reagent is found as Lauryl Sulfate disodium salt (Sigma cat. # L-4390). Dissolve 5.0 g SDS in 25.0 mls of MQ water. In order to mix this reagent properly add a stir bar to the bottle and stir on a hot plate over medium-low heat.

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES		XX		320 mls/tube
NO	XX		XX	

15

- **1.0M MgCl<sub>2</sub>:** Magnesium Chloride

20

Dissolve 20.33g MgCl<sub>2</sub>•6H<sub>2</sub>O (Sigma cat # M-2670) in 80.0 mls of MQ water. Adjust the volume to 100.0 mls total with MQ water.

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES			XX	
NO	XX	XX		XX

25

- **PVPP:** Polyvinylpolypyrrolidone (Sigma cat. # P-6755) for BFB-PCR test. This protocol is taken from Holben *et al* (Appl. Environ. Microbiol. 54:703-711(1988)).

30

1-- Mix 300.0 gms of PVPP in 4 liters of 3.0 M Hydrochloric acid. The HCl. is prepared by diluting concentrated Hcl (12.1N)

2-- Mix and let sit at room temperature in a fume hood for 12-16 hrs with occasional gentle stirring.

3-- Filter sterilized the suspension through Miracloth (Calbiochem, San Diego, CA, Calbiochem cat. #475855). Gentle squeezing of the Miracloth can be done to remove most of the liquid.

Eventually the Miracloth will tear, be careful not to lose any PVPP.

- 4-- Resuspend the PVPP in 4.0 liters of 20 mM Potassium Phosphate buffer, pH=7.4. Prepare the buffer by adding 10.89 gms of  $\text{KH}_2\text{PO}_4$  to a total of 4.0 liters of MQ water. Adjust the pH to 7.4 using 8.0 M KOH (~10 mls).
  - 5-- Soak at room temperature for 1-2 hrs, gently stirring occasionally.
  - 6-- Filter sterilized the suspension through Miracloth as in step 3.
  - 7-- Repeat steps 4 through 6 until the pH of the suspension reaches 7.0 (4 to 5 times).
  - 8-- Filter sterilized through Miracloth as in step 3.
  - 9-- Spread the PVPP out into a thin layer on aluminum foil and dry in a flow hood overnight. Material can be stored in a capped jar and is useful for extended periods of time, >1 year.

10

15

Starting with 300 grams, yield should be ~280 gms after the procedure is completed.

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				0.5 grams/bag
NO	XX	XX	XX	

20

- **Chloroform-Isoamyl Alcohol:** BFB-PCR DNA extraction.

This reagent comes prepared from Sigma (Sigma cat. # C-0549).

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				16.0mls/vial
NO	XX	XX	XX	

25

- **Phenol-Chloroform-Isoamyl Alcohol:** BFB-PCR DNA extraction.  
This reagent comes from Sigma (Sigma cat.# P-2069) with its own equilibration buffer. Add the equilibration buffer to the bottle of Phenol-Chloroform-Isoamyl Alcohol, invert several times to mix, and let the bottle equilibrate in the negative pressure hood for about 2 hours.

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				12.0mls/vial
NO	XX	XX	XX	

- 5        •     **6X Gel loading dye:** To run DNA samples on agarose gels.

This reagent comes prepared from Sigma (Sigma cat. # G-7654).

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				2.0mls/tube
NO	XX	XX	XX	

10

- **Proteinase K:** BFB-PCR and PFGE DNA extraction.

This reagent comes prepared from Sigma (Sigma cat. # P-2308). The reagent comes as a lyophilized powder in 100 mg quantity. Add 5.0 mls of sterile MQ water to the vial, cap tightly, and invert several times to bring the powder to solution. This is a 20 mg/ml stock ready for aliquotting or direct use.

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				63.0mls/tube
NO	XX	XX	XX	

20

- **Isopropanol:** For precipitation of DNA during extraction.

This reagent comes prepared from Sigma (Sigma cat. # I-9516).

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				12.0mls/vial
NO	XX	XX	XX	

25

- **Nusieve 3:1 Agarose:** For restriction endonuclease gel analysis.

This reagent comes from FMC BioProducts, Rockland, ME (FMC Bio Products cat.# 50090).

30

Handling	Micro-waved	U.V. light	filter sterilized	aliquot volume
YES	XX			
NO		XX	XX	XX

- **Seakem LE:** Agarose for analyzing PCR samples.

This reagent comes from FMC BioProducts (FMC BioProducts cat.# 50004).

Handling	Micro-waved	U.V. light	filter sterilized	aliquot volume
YES	XX			
NO		XX	XX	XX

5

- +PCR 100bp low Ladder: A size standard for running agarose gels.  
This reagent comes prepared from Sigma (Sigma cat. # P-1473). In order to dilute the ladder for use locate the concentration of the ladder on the label, for example 102 µg/ml or 102 ng/mµl. The ladder should be run at 50 ng/µl. There is 40µg of total DNA in the tube before dilution. Therefore,  $40\mu\text{g} \div 102\mu\text{g}/\text{ml} = 0.392 \text{ mls}$  or 392 µls of ladder stock and  $102\mu\text{g}/\text{ml} \div 50\mu\text{g}/\text{ml} = 2.04\text{-fold}$  dilution of the ladder stock to reach the proper concentration for use.

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$$2.04 * 392 \mu\text{ls} = 799.68 \mu\text{ls} \text{ or } 800.0 \mu\text{ls} \text{ total volume after dilution.}$$

So,  $800 \mu\text{ls} - 392 \mu\text{ls} = 408 \mu\text{ls}$  this is the total volume of TE + gel loading dye that needs to be added to properly dilute the ladder.

$$800 \mu\text{ls} \div 6 = 133.3 \mu\text{ls} \text{ of gel loading dye (6X concentrate)}$$

$$\text{So, } 392 \mu\text{ls} + 133 \mu\text{ls} = 525 \mu\text{ls}$$

$$\text{and } 800 \mu\text{ls} - 525 \mu\text{ls} = 275 \mu\text{ls} \text{ of TE.}$$

Thus, the final composition of the diluted ladder is 392 µls of ladder stock (i.e. the entire vial) plus 133 µls of 6X gel loading dye plus 275 µls of TE buffer. Aliquot the ladder into 250 µl tubes, approximately 200 µls per tube. Store three tubes at -20°C for long term storage and leave a single tube at 4°C for general use. Use 5 µls per lane on a 2% agarose gel.

Handling	Autoclaved	U.V. light	filter sterilized	aliquot volume
YES				250mls/tube
NO	XX	XX	XX	

30

EXAMPLE 3: Testing of commercial seed lots for the presence of the bacterium *Aac*.

5

The seed in the bags listed below were each subjected to the seed wash protocol described in Example 2a, subjected to DNA extraction as described in Example 2b. PCR was conducted as described in Example 2c. The results are provided below. The results of the PCR and gel electrophoresis are provided in Figures 3a-3c.

Type of Seed: Watermelon. Number of seed per bag: 5,000.

	Batch#	Bag/Sample#s	Vol. PABST/Bag	Final Result
5	628626	1 & 2	600	Both Bags NEG
	628626	3 & 4	600	Both Bags NEG
	628640	5 & 6	600	Both Bags NEG
	540141	7 & 8	600	Both Bags NEG
	628625	9 & 10	600	Both Bags NEG
	628625	11 & 12	600	Both Bags NEG
	619603	13 & 14 - 17	600	Both Bags NEG
	619584	15 & 16 - 18	600	Both Bags NEG
10	Additional Aac Bags #17 & 18	17 & 18		Both Bags POS Check
	Sugar Baby <sup>1</sup>	19& 20	600	NEG (Bag 19) POS (Bag 20)

NEG = NEGATIVE for *Aac*

+200 mls extra

POS = POSITIVE for *Aac*

<sup>1</sup>Control (Commercially Available from Seminis Vegetable Seeds, Inc)

15

Total PABST 6,200 mls.

PABST Buffer	Calls for	Amount weighed
Ascorbic acid (17.6 g/l)	109.12	109.12
Potassium Metabisulfite (2.2 g/l)	13.64	13.64
PH	6.50	6.50

20

Optical Density @ 620nm, Stock *Aac* suspension = 0.107

Colony Counts (48 hours):

Sample #	$10^{-6}$	$10^{-7}$
1	132	11
2	104	13
3	98	7
4	92	6
5	87	9

25

6	110	7
7	82	8
8	102	10
Total	807	71
Average	100.8	8.8

5

Stock population of *Aac* = (Average)(5)(\*Countable dilution) = 1159200 cfu

\_\_\_\_\_ 3864 CFU/ml Seed wash

\_\_\_\_\_ 5654 CFU/gram seed

Spike was made with 2.3 mls of the 10<sup>-3</sup> dilution tube.

10

- The dilution rate which gave bacteria counts between 30 & 300.

#### EXAMPLE 4

This Example describes the random testing of commercial seed lots for the presence of the bacterium *Aac*.

Type of Seed: Watermelon. Number of seed per bag: 5,000.

15

The seeds in the bags listed below were each subjected to the seed wash protocol described in Example 2a, subjected to DNA extraction as described in Example 2b. PCR was conducted as described in Example 2c. The results of such analysis is provided below. The results of the PCR and gel electrophoresis are provided in Figures 4a-4c.

20

Batch#	Bag/Sample #s	Vol. PABST/Bag	Final Result
583268	1 & 2	600	Both Bags NEG
583268	3 & 4	600	Both Bags NEG
634949	5 & 6	600	Both Bags NEG
634949	7 & 8	600	Both Bags NEG
628640	9 & 10	600	Both Bags NEG
628640	11 & 12	600	Both Bags NEG
540141	13 & 14	600	Both Bags NEG
540141	15 & 16	600	Both Bags NEG
583266	17 & 18	600	Both Bags NEG
Sugar Baby <sup>1</sup>	19 & 20	600	NEG Bag (19) POS Bag (20)

25

30

NEG = NEGATIVE for *Aac*

+200 mls extra

POS = POSITIVE for *Aac*

<sup>1</sup>Control

Total PABST 6,200 mls

PABST Buffer	Calls for	Amount weighed
Ascorbic acid (17.6 g/l)	109.12	109.12
Potassium Metabisulfite (2.2 g/l)	13.64	13.64
PH	6.50	6.50

5 Optical Density @ 620nm, Stock *Aac* suspension = 0.103

Colony Counts (48 hours):

Sample #	$10^{-6}$	$10^{-7}$
1	60	4
2	59	3
3	67	6
4	55	7
5	72	8
6	54	6
7	62	7
8	57	10
Total	486	51
Average	60.75	6.37

Stock population of *Aac* =(Average)(5)(\*Countable dilution)= 699200 cfu

20        2330 CFU/ml Seed wash

3410 CFU/gram seed

Spike was made with 2.3 mls of the  $10^{-3}$  dilution tube.

\* The dilution rate which gave bacteria counts between 30 & 300.

EXAMPLE 5: Testing of commercial seed lots for the presence of the

25        bacterium *Aac*.

The seed in the bags listed below were each subjected to the seed wash protocol described in Example 2a, subjected to DNA extraction as described in Example 2b. PCR was conducted as described in Example 2c. The results are provided below. The results of the PCR and gel electrophoresis are provided in 30 Figures 5a-5c.

Type of Seed: Watermelon. Number of seed per bag: 5,000.

Batch #	Bag/Sample #s	Vol. PABST/Bag	Final Result
539379	1 & 2	600	Both Bags NEG
610419	3 & 4	600	Both Bags NEG
610419	5 & 6	600	Both Bags NEG
636115	7 & 8	600	Both Bags NEG
636115	9 & 10	600	Both Bags NEG
616997	11 & 12	600	Both Bags NEG
616997	13 & 14	600	Both Bags NEG

628627	15 & 16	600	Both Bags NEG
628627	17 & 18	600	Both Bags NEG
Sugar Baby <sup>1</sup>	19 & 20	600	NEG (Bag 19) POS (Bag 20)

NEG = NEGATIVE for *Aac*

+200 mls extra

5 POS = POSITIVE for *Aac*

<sup>1</sup>Control

Total PABST 6,200 mls

PABST Buffer	Calls for	Amount weighed
Ascorbic acid (17.6 g/l)	109.12	
Potassium Metabisulfite (2.2 g/l)	13.64	
PH	6.50	

Optical Density @ 620 nm, Stock *Aac* suspension = 0.105

Colony Counts (48 hours):

	Sample #	$10^{-6}$	$10^{-7}$
15	1	107	6
	2	82	9
	3	89	9
20	4	73	11
	5	91	9
	6	97	10
	7	69	7
	8	84	7
25	Total	692	68
	Average	86.5	8.5

Stock population of *Aac* = (Average)(5)(\*Countable dilution) = 993600 cfu

3312 CFU/ml Seed wash

4846 CFU/gram seed

Spike was made with 2.3 mls of the  $10^{-3}$  dilution tube.

30 \* The dilution rate which gave bacteria counts between 30 & 300.

EXAMPLE 6: Testing of commercial seed lots for the presence of the bacterium *Aac*.

35 The seed in the bags listed below were each subjected to the seed wash protocol described in Example 2a, subjected to DNA extraction as described in Example 2b. PCR was conducted as described in Example 2c. The results are

provided below. The results of the PCR and gel electrophoresis are provided in Figures 6a-6c.

Type of Seed: Watermelon. Number of seed per bag: 5,000.

Batch#	Bag/Sample #s	Vol. PABST/Bag	Final Result
539379	1 & 2	600	Both Bags NEG
539379	3 & 4	600	Both Bags NEG
507037	5 & 6	600	Both Bags WEAK POS
479086	7 & 8	600	Both Bags WEAK POS
641699	9 & 10	600	Both Bags NEG
641696	11 & 12	600	Both Bags NEG
574454	13 & 14	600	Both Bags WEAK POS
151342	15 & 16	600	Both Bags WEAK POS
561864	17 & 18	600	Both Bags NEG
Sugar Baby <sup>1</sup>	19 & 20	600	NEG (Bag 19) POS (Bag 20)

NEG = NEGATIVE for *Aac*

+200 mls extra

POS = POSITIVE for *Aac*

<sup>1</sup>Control

Total PABST 6,200 mls

PABST Buffer	Calls for	Amount weighed
Ascorbic acid (17.6 g/l)	109.12	109.12
Potassium Metabisulfite (2.2 g/l)	13.64	13.64
PH	6.50	6.50

Optical Density @ 620nm, Stock *Aac* suspension = 0.102

Colony Counts (48 hours):

Sample #	10 <sup>-6</sup>	10 <sup>-7</sup>
1	47	11
2	80	10
3	52	10
4	57	6
5	60	5
6	71	8
7	63	5
8	70	4

Total	500	59
Average	62.5	7.37

Stock population of *Aac* = (Average)(5)(\*Countable dilution)=717600 cfu

5        2392 CFU/ml Seed wash

3500 CFU/gram seed

Spike was made with 2.3 mls of the 10<sup>-3</sup> dilution tube.

- The dilution rate which gave bacteria counts between 30 & 300.

EXAMPLE 7: Testing of commercial seed lots for the presence of the bacterium *Aac*.

10      The seed in the bags listed below were each subjected to the seed wash protocol described in Example 2a, subjected to DNA extraction as described in Example 2b. PCR was conducted as described in Example 2c. The results are provided below. The results of the PCR and gel electrophoresis are provided in Figures 7a-7c.

15      Type of Seed: Watermelon. Number of seed per bag: 5,000.

Batch#	Bag/Sample #s	Vol. PABST/Bag	Final Result
640209 <sup>2</sup>	1 & 2	600	Both Bags NEG
520272	3 & 4	600	Both Bags POS
537894	5 & 6	600	Both Bags POS
559750	7 & 8	600	Both Bags NEG
559750	9 & 10	600	Both Bags NEG
532146	11 & 12	600	Both Bags POS
518847	13 & 14	600	Both Bags NEG
518773	15 & 16	600	Both Bags POS
506797	17 & 18	600	Both Bags POS
Sugar Baby <sup>1</sup>	19 & 20	600	NEG (Bag 19) POS (Bag 20)

NEG = NEGATIVE for *Aac*

+200 mls extra

POS = POSITIVE for *Aac*

<sup>1</sup>Control

30      <sup>2</sup>Bags 1 & 2 combined contained 6,685 seeds

Total PABST 6,200 mls

PABST Buffer	Calls for	Amount weighed
Ascorbic acid (17.6 g/l)	109.12	109.12
Potassium Metabisulfite (2.2 g/l)	13.64	13.64

PH	6.50	6.51
----	------	------

Optical Density @ 620nm, Stock *Aac* suspension =.097

Colony Counts (48 hours):

	Sample #	$10^{-6}$	$10^{-7}$
5	1	113	9
	2	97	13
	3	91	11
10	4	120	13
	5	89	7
	6	106	9
	7	84	10
	8	94	12
15	Total	794	84
	Average	99.2	10.5

Stock population of *Aac* =(Average)(5)(\*Countable dilution)=1140800 cfu

\_3802\_\_\_\_\_ CFU/ml Seed wash

\_5564\_\_\_\_\_ CFU/gram seed

20 Spike was made with 2.3 mls of the  $10^{-3}$  dilution tube.

\* The dilution rate which gave bacteria counts between 30 & 300.

EXAMPLE 8: Testing of commercial seed lots for the presence of the bacterium *Aac*.

25 The seed in the bags listed below were each subjected to the seed wash protocol described in Example 2a, subjected to DNA extraction as described in Example 2b. PCR was conducted as described in Example 2c. The results are provided below. The results of the PCR and gel electrophoresis are provided in Figures 8a-8c.

Type of Seed: Watermelon. Number of seed per bag: 5,000.

30	Batch#	Bag/Sample #s	Vol. PABST/Bag	Final Result
	539444	1 & 2	600	Both Bags WEAK POS
	355992	3 & 4	600	Both Bags WEAK POS
	539733	5 & 6	600	Both Bags WEAK POS
	507080	7 & 8	600	Both Bags Both

			Bags WEAK POS
35	513097	9 & 10	600 Both Bags NEG
	641699	11 & 12	600 Both Bags NEG
	641699	13 & 14	600 Both Bags NEG
	641696	15 & 16	600 Both Bags NEG
	641696	17 & 18	600 Both Bags NEG
40	Durango (298675) <sup>1</sup>	19 & 20	600 NEG (Bag 19) POS (Bag 20)

NEG = NEGATIVE for *Aac*

+200 mls extra

POS = POSITIVE for *Aac*

<sup>1</sup>Control (Commercially Available from Seminis Vegetable Seeds, Inc.)

Total PABST 6,200 mls

PABST Buffer	Calls for	Amount weighed
Ascorbic acid (17.6 g/l)	109.12	109.14
Potassium Metabisulfite (2.2 g/l)	13.64	13.64
PH	6.50	6.52

Optical Density @ 620nm, Stock *Aac* suspension = 0.107

50

Colony Counts (48 hours):

	$10^{-6}$	$10^{-7}$
5	107	6
	87	9
	71	9
	83	4
	98	3
10	76	4
	79	6
	85	5
	Total	46
	Average	85.7
		5.75

Stock population of *Aac* = (Average)(5)(\*Countable dilution)=986700 cfu

15        3289        CFU/ml Seed wash

4813        CFU/gram seed

Spike was made with 2.3 mls of the  $10^{-3}$  dilution tube.

\* The dilution rate which gave bacteria counts between 30 & 300.

EXAMPLE 9: Testing of commercial seed lots for the presence of the

20        bacterium *Aac*.

The seed in the bags listed below were each subjected to the seed wash protocol described in Example 2a, subjected to DNA extraction as described in Example 2b. PCR was conducted as described in Example 2c. The results are provided below. The results of the PCR and gel electrophoresis are provided in Figures 9a-9c.

25        Type of Seed: Watermelon. Number of seed per bag: 5,000.

Batch#	Bag/Sample #s	Vol. PABST/Bag	Final Result
634949	1 & 2	600	Both Bags NEG
610418	3 & 4	600	Both Bags NEG
610418	5 & 6	600	Both Bags NEG
501273	7 & 8	600	Both Bags NEG
501273	9 & 10	600	Both Bags NEG
636115	11 & 12	600	Both Bags NEG
610420	13 & 14	600	Both Bags NEG
610420	15 & 16	600	Both Bags NEG
583268	17 & 18	600	Both Bags NEG
Sugar Baby <sup>1</sup>	19 & 20	600	NEG (Bag 19) POS (Bag 20)

NEG = NEGATIVE for *Aac*

+200 mls extra

POS = POSITIVE for *Aac*

<sup>1</sup>Control

Total PABST 6,200 mls

5	PABST Buffer	Calls for	Amount weighed
	Ascorbic acid (17.6 g/l)	109.12	109.12
	Potassium Metabisulfite (2.2 g/l)	13.64	13.64
	PH	6.50	6.51

Optical Density @ 620nm, Stock *Aac* suspension = 0.102

10 Colony Counts (48 hours):

	Sample #	$10^{-6}$	$10^{-7}$
15	1	81	9
	2	107	6
	3	91	5
	4	110	11
	5	79	6
	6	74	7
	7	86	6
20	8	113	7
	Total	741	57
	Average	92.6	7.1

Stock population of *Aac* =(Average)(5)(\*Countable dilution)= 1064900 cfu

25      3549 CFU/ml Seed wash

5194 CFU/gram seed

Spike was made with 2.3 mls of the  $10^{-3}$  dilution tube.

- The dilution rate which gave bacteria counts between 30 & 300.

## EXAMPLE 10

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This Example provides a comparison of specificity of two different primer pairs (RST 90/91 and SEQ ID NOS: 1-2). Comparisons were made with isolates of *Acidovorax* spp. and numerous other bacterial isolates. All DNA was prepared using the 'boil prep' method which consists of making a suspension of the bacteria in 200 ul of water in a 1.5 ml Eppendorf tube and placing the tubes in a boiling water bath for about 10 minutes. Data is a compilation of two different experiments (PCR #71 and #169). ND=not done.

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	Bacterial Isolate	PCR response of two different primer sets	
		RST 90/91	SEQ ID NOS:1-2
Pseudomonas syringae	pv. <i>tomato</i> A	-	-
	" " " B	-	-
<i>Pseudomonas syringae</i>	pv. <i>lachrymans</i> A	-	-
	" " " B	-	-
<i>Clavibacter michiganensis</i>	pv. <i>michiganensis</i> A	-	-
	" " " B	-	-
<i>Xanthomonas campestris</i>	pv. <i>vesicatoria</i> A	-	-
	" " " B	-	-
<i>Acidovorax avenae</i>	pv. <i>citrulli</i> 154-Indiana	90	1
	" " " 163-Guatemala	0	0
	" " " 107-Texas(citron)	ND	0
	" " " 170-Texas	ND	0
	" " " 111-Georgia	ND	0
	" " " 12-S. Carolina	ND	0
	" " " 7500, New Zealand	ND	0
	" " " 37 D. Antibiotic Mut.	ND	0
	" " " 6 other isolates	ND	6
	<i>Comomonas acidovorans</i>	0	-
<i>Xanthomonas campestris</i>	pv. <i>carota</i>	ND	-
	pv. <i>vitiens</i>	ND	-
<i>Acidovorax avenae</i>	pv. <i>avenae</i>	+ <sup>1</sup>	6
	pv. <i>cattylae</i>	+ <sup>1</sup>	0
<i>Acidovorax konjacii</i>		+ <sup>1</sup>	0
	<i>Acidovorax facilis</i>	- <sup>1</sup>	-
<i>Hydrenophaga</i> sp. "H bacterium"		- <sup>1</sup>	+(weak)

<sup>1</sup> Indicates the response reported in ASRF grant reports from Stall and Kucarek, Dec. 1994.

40

## EXAMPLE 11

In this example, four different sources of seed (2500 seed/sample) were grown in the greenhouse and evaluated for BFB to determine the percent transmission (%Trans). Different samples of the same seed sources (2500 seed/sample) were taken through the BFB seed assay and the final seed extract was separated into two samples. One sample was plated onto semiselective media (NBC) and the other was taken through the DNA extraction protocol (described in Example 1) and was subjected to PCR using two different primer sets. After two days of growth the NBC plates were sampled and subjected to PCR. Additional isolations were made to recover *Acidovorax*. The PCR response is expressed as positive (+) or negative (-). The intensity of the gel band on the positives is rated from low (+) to high (+++). All samples were tested undiluted and diluted 1:10.

15	Sample Label	% Trans.	PCR responses							
			RST 90/91				SEQ ID NOS:1-2			
			Seedwash		NBC		Seedwash		NBC	
			10 <sup>0</sup>	10 <sup>-1</sup>	PCR	Isol.	10 <sup>0</sup>	10 <sup>-1</sup>	PCR	Isol.
20	"Low A"	0.08	-	+	-	-	+	+++	-	-
	"Low B"	0.12	++	++	-	-	+++	+++	-	-
	"Hi B"	8.34	+++	+++	-	-	+	+++	+(faint)	-
	"Check"	0.00	-	-	-	-	-	-	-	-

## EXAMPLE 12

This Example shows the effects of diluting the extracted DNA prior to running the material through a BioRad Sephadex column on the ability of the PCR to detect the presence of *Acidovorax avenae* pv *citrulli*. A dilution series (10 fold) was made from an original suspension of *Aac* that was 0.1 OD 620<sub>nm</sub>. Specific amounts were added to samples of 5,000 Charleston Grey watermelon seed. Further dilutions were plated onto Nutrient Agar and counts were made to determine the amount of cells added to the samples. Values are expressed as Colony Forming Units (CFU)/ml of seedwash suspension. Results are expressed as the number of positive PCR reactions over the total number of reactions.

### PCR #208:

Dilution factor prior to running a column	Number of Positive PCR Reactions over the total number of reactions at differing concentrations of Aac.			
	0 CfU/ml	1030 CfU/ml	5130 CfU/ml	25,700 CfU/ml

Undiluted	0/4	0/8	0/8	0/8
1:50	0/4	6/8	8/8	8/8
1:100	0/4	3/8	6/8	8/8

5 EXAMPLE 13

This example provides a comparison of the sensitivity of two different primer pairs (RST 90/91 and SEQ ID NOS: 1-2) in the detection of *Acidovorax* DNA from various sources at differing concentrations. Data is compiled from several different experiments.

	PCR #	DNA Source	Lower limit of concentration of DNA yielding a positive PCR response		
			RST 90/91	SEQ ID NOS:1-2	Ratio RST/SEQ ID NOS:1-2
15	56A	Culture boil prep	0.8 ng	0.2 ng	5
		Culture boil prep-older	20.0 ng	0.8 ng	25
		Purified DNA-culture	160.0 pg	6.4 pg	25
20	57	Purified Culture DNA	3.2 pg	0.02 pg	125
	59	Purified Culture DNA	400.0 pg	0.6pg	625
25	70	Purified Culture DNA	20.0 pg	2.0 pg	10
	73	Purified Culture DNA	100.0 pg	10.0 pg	10
Range			3.2-800.0 pg	0.03-160.0pg	5-625

#### EXAMPLE 14

This Example provides a summary of several experiments showing the sensitivity of the PCR test in the detection of different concentrations of *Acidovorax avenae* pv *citrulli*. Samples of 5,000 watermelon seed were spiked with different amounts of bacteria. A dilution series (10 fold) was made from an original suspension of *Aac* that was 0.1 OD<sub>620<sup>nm</sup></sub> and specific amounts were added to the seed samples. Further dilutions were plated onto Nutrient Agar and counts were made to determine the amount of cells added to the samples. Values are expressed as Colony Forming Units (CFU)/ml of seed-wash suspension. All experiments were done using the primers in SEQ ID NOS: 1-2 except Experiment 8 which included both RST 90/91 and the primers shown in SEQ ID NOS: 1-2. Results are expressed as the number of positive PCR reactions over the total number of PCR reactions run.

Experiment 1 (PCR 247)		Experiment 2 (PCR 252)		Experiment 3 (PCR 261)		Experiment 4 (PCR 267)		Experiment 5 (PCR 268)	
CFU/ml	#Pos/tot								
0	0/6	0	0/4	0	0/4	0	0/4	0	0/4
624	12/12	25	5/8	159	8/8	301	8/8	191	8/8
1,293	12/12	123	8/8	616	8/8	904	8/8	574	8/8
3,879	12/12	247	8/8	1232	8/8	1804	6/6	1148	8/8

  

Experiment 6 (PCR 275)		Experiment 7 (PCR 355)		Experiment 8-RST 90/91 primers (PCR 362)		Experiment 8-SEQ ID NO:1-2 (PCR 362)	
CFU/ml	#Pos/tot	CFU/ml	#Pos/tot	CFU/ml	#Pos/tot	CFU/ml	#Pos/tot
0	0/4	0	0/4	0	0/4	0	0/4
333	8/8	678	8/8	29	0/6	29	3/6
1000	8/8	1696	6/8	59	0/6	59	2/6
2000	8/8	3392	8/8	133	0/6	133	0/6
				590	0/6	590	6/6

## EXAMPLE 15

This example demonstrates the detection of *Acidovorax avenae* subsp. *citrulli* in watermelon seed samples (5,000 seed/sample) spiked with naturally infected seed and broth cultures. One sample of each variety was spiked with ten (10) seed of a BFB naturally infected seedlot. A second sample was spiked with a specific volume of a bacterial suspension. A dilution series (10 fold) was made from an original suspension of *Aac* that was 0.1 OD  $620_{\text{nm}}$ . Specific amounts were added to samples of 5,000 seed and further dilutions were plated onto Nutrient Agar and counts were made to determine the amount of cells added to the samples. Values are expressed as Colony Forming Units (CFU)/ml of seed-wash suspension. The PCR response is expressed as positive (+) or negative (-). The intensity of the gel band on the positives is rated from low (+) to high (+++).

Variety	Spike material	CFU/ml	PCR Response	
			RST 90/91	SEQ ID NOS:1-2
Charleston Grey 133*	Single seed	--	+(faint)	++
	Culture	1,286	-	+
Royal Charleston WR*	Single seed	--	+	+++
	Culture	2,214	-	++
Charlee*	Single seed	--	+	+++
	Culture	1,185	-	+
EH1952*	Single seed	--	+	+++
	Culture	3,973	-	+
Picnic*	Single seed	--	-	+
	Culture	2,392	-	+ (Very Faint)
Sabrina*	Single seed	--	+	+++
	Culture	3,570	-	++
Starbrite*	Single seed	--	+(faint)	++
	Culture	1,392	-	+
Sugarbrite*	Single seed	--	+	+++
	Culture	3,164	-	+
Sugar Baby*	Single seed	--	+	+++
	Culture	3,131	-	+
#Pos/Total			8/18	18/18

\*Commercially Available from Seminis Vegetable Seeds, Inc.

## EXAMPLE 16

This example provides a comparison of PCR versus grow-out test results in detection of *Acidovorax avenae* pv. *citrulli*. Thirty samples of 3300 watermelon seed each were obtained as blind samples. Half of the samples were grown the in greenhouse for 18 days and the other half were tested by the direct seed PCR assay. After all results were obtained the previous test result information was given. Results are expressed as the number of positive lots over the total number of lots tested.

### PCR #757:

Test Method	# seedlots positive for Aac /total number tested (Each column heading represents the previous three test results obtained for the samples)		
	+++	-++	---
GH Growout	5/5	4/5	0/5
PCR Seed assay	5/5	3/5 <sup>1</sup>	0/5

<sup>1</sup>All five of the samples yielded unusual DNA pellets; the pellets would not resuspend into solution. After physically breaking up the pellets, three samples were found positive in the PCR test. The other two samples were spiked with known Aac DNA and run in the PCR. The test was positive indicating that no inhibitor was present. This also suggests that the DNA was bound by the pellet and rendered unavailable for a PCR reaction to occur.

## EXAMPLE 17

Example 17 provides a comparison of PCR versus grow-out test results in detection of *Acidovorax avenae* pv. *citrulli* in watermelon seed. Individual seed of a known naturally infected seed lot were planted in pots or flats and grown out under greenhouse conditions conducive for disease expression. All pots and flats were monitored daily for disease symptoms and any plants showing symptoms were confirmed by plant tissue PCR. In addition, single seeds of the same source were added to samples of 5,000 known healthy watermelon seed and run through the direct seed PCR assay. Results are expressed as the percent of BFB in the samples.

	Test Method	Percent Germination (Infected seed source)	Percent BFB (#Pos/Total)
5	<p>GH Growout:</p> <p>Expt 1—1 infected watermelon seed/pot, pots covered with plastic bags, area covered with shade cloth.</p> <p>--- 1 infected watermelon seed/pot, pots uncovered, and normal greenhouse conditions.</p>	96.4	30.3 (73/241)
10	<p>Expt 2—1 infected watermelon seed per pot, pots uncovered, and normal greenhouse conditions.</p> <p>Expt 3—One infected watermelon seed per flat with 200 healthy seed, normal greenhouse conditions.</p>	92.8 93.6	18.5 (43/232) 9.4 (22/234)
15		95.0	8.4 (20/237)
20	<p>PCR direct seed assay:</p> <p>Expt 1---1 infected watermelon seed in 5,000 healthy seed of Sugar Baby.</p> <p>Expt 2---One infected watermelon seed in 5,000 healthy seed of Charleston Gray.</p>	-NA-	100 (16/16) 100 (16/16)

All references referred to herein are hereby incorporated by reference.

The present invention is illustrated by way of the foregoing description and examples. The foregoing description is intended as a non-limiting illustration, since many variations will become apparent to those skilled in the art in view thereof. It is intended that all such variations within the scope and spirit of the appended claims be embraced thereby.

Changes can be made to the composition, operation, and arrangement of the method of the present invention described herein without departing from the concept and scope of the invention as defined in the following claims.